Selective Cell Culture of Primary Breast Carcinoma

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Abstract

We have used culture conditions which simulate the microenvironment of breast tumors for the isolation and propagation of primary breast tumor cells in vitro. In this monolayer setup, the mixture of cells dissociated from primary breast tumors is subjected to self-created gradients of oxygen and nutrients as well as metabolic waste and extracellular pH. The tumor populations isolated under these novel conditions have displayed phenotypic properties characteristic of breast carcinomas, including homogeneous expression of cytokeratin 19, and increased mitochondrial retention of the cationic dye rhodamine 123. Nonmalignant cultures from reduction mammoplasty were unable to survive these conditions. One tumor population which reached passage 10 was aneuploid for chromosomes 15 and 17, and displayed a p53 mutation in exon 8. These studies strongly suggest that the culture conditions described here can suppress the growth of normal breast cells, thereby allowing selective isolation of some populations of slow-growing primary tumor cells in vitro.

Introduction

Cell culture models established directly from patient tumors could be an important renewable resource for examining and manipulating relevant molecular and cellular changes underlying malignant breast disease. In achieving this goal, a major challenge is presented by the histopathological architecture of this lesion. Typically, malignant specimens consist of cords or nests of tumor cells invading neighboring stroma and thereby becoming closely juxtaposed to apparently nonmalignant areas of the tissue. Thus, separation of tumor cells from nonmalignant epithelium for propagation in vitro becomes problematic. Limitations in this regard have included: (a) the lack of clear morphological distinction between malignant and nonmalignant cultures, and (b) the slow proliferation rate of primary breast tumor cells resulting in the overgrowth of nonmalignant epithelium. It is not surprising therefore that when primary tumor specimens are dissociated and propagated under routine conditions, the resulting culture is quite normal in appearance and chromosomal content (1, 2). Only a few sporadic instances have been reported in which primary tumor specimens have yielded long-term cultures representing the original breast tumor (3–5). Our novel approach described here takes into account the fact that a suboptimal, nutritionally depleted environment exists in breast tumors. This is evident as necrotic areas in cross-sections of the tumor even during preinvasive stages such as carcinoma in situ (6). Cell necrosis in tumors is often due to inadequate vascular supply. In this regard, studies on vascularization of human tumors have shown that the preexisting normal host vasculature can fulfill microcirculatory functions only for short time periods in the newly emerging tumor. Structural and functional changes soon begin to occur, which can result in a hostile metabolic environment in certain areas of the lesion (7). Given these suboptimal conditions, how then do tumors proliferate, invade, and proceed to kill the host? It is widely hypothesized that tumor cell populations whose intrinsic characteristics enable their growth to be autonomous, or relatively independent of this nutritionally depleted environment are the progenitors from which more aggressive phenotypes continue to evolve. Oftentimes, normal cells do not survive such nutritional constraints. It is conceivable that the majority of tumor cells may also be sensitive to extreme deprivation, and cell survival may indeed be dependent on the relative location within a nutritional gradient. On the basis of this hypothesis, we have adopted a microenvironment in which selective isolation and propagation of tumor cells is feasible. The isolated cultures express phenotypes ascribed to tumor cells.

Materials and Methods

Specimen Dissociation. Five specimens of infiltrating ductal breast carcinoma and two specimens of nonmalignant reduction mammoplasty tissue were mechanically and enzymatically dissociated to yield clumps of epithelial cells termed organoids, as described earlier (8). Organoids from tumor tissue were trypsin dissociated to single cells and washed with serum-containing medium before seeding in culture. Epithelial cells from reduction mammoplasty specimens were derived from partial trypsinization of organoid outgrowths as described previously (8). Cells were propagated at 37°C in 0.1% CO2-air. In serum-free, growth factor-supplemented medium MCDB 170 (Clonetics, San Diego, CA) containing 0.06 mM calcium. A reduced calcium concentration was used in an effort to prolong the proliferation potential of both malignant and nonmalignant cells in culture, thereby providing greater cell numbers for phenotypic analyses. All dissociated material was assigned passage 1 upon first seeding in culture.

SWC. The SWC setup used in this study was first described by Hlatky and Alpert (9), and previously used for mammary epithelial cell culture (10). Briefly, two clean, sterile glass microscope slides were placed side by side in a 3.5- x 3.5-inch Integrid Petri dish (Falcon). Custom-designed plexiglass holders were used to prevent the slides from moving around in the dish. A single-cell suspension of 2 x 103 cells/dish was seeded in 12 ml of growth medium onto the slides. After allowing 24 h for cell attachment and spreading, 150-μm sterile glass spacers were placed at each end of the slide, and a top slide was laid down on the spacers so that the cell monolayer was completely covered from the top. This setup is called a SWC. For renewal of growth medium, the SWC was “unsandwiched” by lifting off the top slide with sterile forceps. Characterization of SWC-isolated tumor cells was performed after the initial isolates were expanded and passed under routine culture conditions.

Immunostaining. Fourth passage SWC-isolated cells growing in 35-mm tissue culture dishes were fixed for 10 min with 1:1 ethanol/aceton and used for immunofluorescence with anti-pan CK (Enzo Labs, New York, NY), or monospecific anti-C3B19, clone Ks 19.1 (Progen, Heidelberg, Germany). Biotinylated antimouse antibody (Vector Laboratories, Burlingame, CA) followed by fluoresceinated-ultra avidin (Leinco, St. Louis, MO) were used for signal amplification and detection.

1 The abbreviations used are: SWC, sandwich culture; R123, rhodamine 123; SSCP, single-strand conformational polymorphism; CK, cytokeratin.
R123 Retention. Uptake and retention of R123 (Eastman Kodak, Rochester, NY) was measured by incubating monolayers with 10 µg/ml R123 in growth medium for 30 min. Cultures were rinsed and refed with R123-free medium. Twenty-four h later, retention was observed microscopically under an epifluorescent, FITC illumination system and recorded photographically. To eliminate the possibility that R123 retention was merely a transient, SWC-induced effect, the samples that were analyzed for this phenotype were maintained under routine culture conditions for two additional passages after exposure to SWC.

Fluorescence in Situ Hybridization. Aneuploidy in interphase nuclei of cultured cells was measured as described previously (11). Pericentromeric probes to chromosomes 15 and 17 were used on fixed monolayers. Cytospin cultured cells was measured as described previously (11). Pericentromeric culture conditions for two additional passages after exposure to SWC.

Results

We have adopted culture conditions which simulate the in vivo milieu of breast carcinoma by using a two-dimensional, multicellular in vitro model called SWC for the selective isolation of primary breast tumor cells. In a modification of the setup first described by Hlatky and Alpen (9), anchorage-dependent cells from dissociated primary tumor tissue are “sandwiched” between two glass microscope slides separated by a narrow gap. A thin layer of growth medium covers the cells and fills the narrow gap between the slides, and as the SWC ages, it becomes a self-created gradient of oxygen, nutrients, pH, and metabolic waste (Fig. 1). Other useful features of the modified SWC setup are that each dish contains two “sister sandwiches” and an unsandwiched area comprising one-third of the culture dish. This allows (a) the assessment of results from replicate sandwiches subjected to the variables within a single culture dish, and (b) the unsandwiched area serves as a control representing routine cell culture conditions.

Cryopreserved organoids isolated from five specimens of primary infiltrating ductal carcinoma were trypsin dissociated to single cells and plated in SWC. Cultures were left undisturbed for 7 days. At this point, the top slide was removed, and the cultures were unsandwiched and replenished with fresh medium to allow cell recovery. Microscopically, sandwiched cells appeared “sick” and quiescent. Considerable cell lysis was observed in the most depleted, central region of the SWC. After 7 days of recovery, during which the cultures were fed at 2–3-day intervals, small, healthy clonal patches of 8–16 cells were observed, approximately 1–2/SWC. Two weeks later, these patches were “resandwiched” for 7 days. At the end of this period, a few patches had disintegrated, but the majority appeared to be unchanged. From this point on, cultures were maintained under routine conditions. Cell proliferation was soon resumed, resulting in relatively large patches of cuboidal epithelial cells within 2–4 weeks, which were eventually allowed to grow as mass cultures. These SWC isolates of primary tumor specimens were examined for the presence of CK19.

All five specimens showed homogeneous expression of this phenotype (Fig. 2a). All five specimens also displayed increased retention of the fluorescent, lipophilic dye R123 when compared to cultures of nonmalignant human mammary epithelial cells (Fig. 2, b and c).

In SWCs of human mammary epithelial cells derived from nonmalignant reduction mammaplasty tissue, some cells were attached to the substrate only in a very narrow rim at the edges of the sandwches, whereas in all other areas, cells had disintegrated. Microscopically observable growth resumption was not seen in the adherent cells of nonmalignant SWCs. Additionally, reconstruction experiments were set up in SWC where nonmalignant human mammary epithelial cells were mixed at a ratio of 1000:1 with morphologically distinct cells of the benzo(a)pyrene-immortalized cell line 184B5 (8). In these experiments, small colonies of the 184B5 cell line were present throughout the culture, which resumed rapid growth upon unsandwiching, whereas nonmalignant cells were growth arrested or had detached and disintegrated (data not shown).

From the findings described above, it is evident that by using the SWC setup, we have isolated epithelial cells from tumor tissue which are indeed distinct from cultures of nonmalignant epithelium. However, unlike established cell lines, these cultures are not immortalized. Consequently, SWC isolates from tumor tissue could only be passaged a few times. In all cases of carcinoma-derived cells, upon initial seeding into SWC, the culture was assigned passage 1. Three of five specimens could not be propagated beyond the fourth passage, one beyond the fifth passage, while one specimen was passaged 10 times. The population doubling time in all cultures was relatively long, allowing a 1:1 split at mid-confluence every 2–3 weeks. The tumor specimen that was most prolific after isolation from SWC and was passaged 10 times had a doubling time of 7 days.

Fluorescence in situ hybridization analysis of SWC-isolated primary tumor cells at passage 6 with pericentromeric probes showed that over 60% of the cells were aneuploid for chromosomes 15 and 17, with 3–7 copies/cell (Fig. 2d). It is quite possible that the remainder, which were diploid with these probes, were aneuploid at other...
Fig. 2. Phenotype of SWC-isolated tumor cells. a, indirect immunofluorescence of CK19 in SWC-isolated breast tumor cells. Note homogeneous staining of entire culture. b, R123 fluorescence in primary tumor cells isolated from SWC. Note considerable dye retention in cells after 24-h incubation in dye-free medium compared to nonmalignant human mammary epithelial cell culture shown in c. Weak fluorescence in c demonstrates inability of nonmalignant cells to retain dye. Conditions of dye exposure, fluorescence microscopy, and photography are identical in b and c. d, aneusomy for chromosome 17 displayed as multiple signals/cell using fluorescence in situ hybridization analysis with a pericentromeric probe. Nuclei counterstained with 4,6-diamidino-2-phenylindole are shown in d'.

Fig. 3. p53 mutation analysis in primary tumor cells isolated from SWC. DNA was amplified by PCR with primers 8U and 8D (12) and subjected to direct double-stranded DNA sequencing. Right of each panel, wild-type and mutated codons in exon 8.

Discussion

Experimental models such as multicellular spheroids are widely used to simulate the microecology of solid tumors and applied toward an improved understanding of tumor biology (13). However, an important prerequisite for spheroid formation is for the tumor cells to exhibit anchorage-independent growth in suspension culture. Specifically with regard to breast cancers, primary tumor cells do not proliferate in the absence of a solid substrate and growth in suspension is only rarely observed in those advanced metastatic specimens which develop into established cell lines. More recently, Petersen et al. (14) have reported on the formation of small, multicellular colonies of normal and malignant breast epithelial cells embedded in reconstituted basement membrane. In this system, interesting phenotypic differences between colonies of normal and malignant cells were observed which may be potentially useful in the selective isolation of tumor cells. These phenotypic differences, however, appear to be associated with the ability of tumor cells to escape basement membrane-induced differentiation signals; generally a feature of more advanced malignant stages also. In contrast, the two-dimensional, monolayer sandwich culture system that we have adopted in this study for the selective growth of primary tumors mimics a microenvironment often observed even in preinvasive lesions, and may therefore be particularly relevant toward the isolation of cells displaying relatively early changes in cancer.

We have demonstrated here that in the SWC system, a population of tumor-derived epithelial cells have survived conditions of nutrient...
and oxygen deprivation and subsequently resumed growth in vitro, whereas reduction mammoplasty-derived epithelial cells are irreversibly damaged and do not yield proliferating populations. In addition to identifying optimal conditions for the selective isolation and growth of primary tumor cells, we have demonstrated that tumor-derived cells isolated using this novel method display characteristics associated with malignant cells. Tumor-derived SWC isolates homogeneously resemble the original tumor. Since established breast carcinoma cell lines, when isolated from primary tumors, did not display any characteristics of the major basal epithelial CKs, 5 and 14 (15, 16). In contrast to SWC-isolated cells, anti-CK19-positive cells initiated under routine serum-free culture conditions from both nonmalignant and primary breast carcinoma tissue do not maintain proliferation and are rapidly eliminated (16). In a recent study, improved growth conditions were reported which support proliferation of the CK19-expressing luminal subset (17). However, these CK19-expressing cells, even when isolated from primary tumors, did not display any characteristics of the original tumor. Since established breast carcinoma cell lines continue to grow in culture while expressing CK19, the presence of this phenotype in a putative primary tumor cell population serves as a good indicator of authenticity, particularly when the expression of other normal phenotypes has been ruled out. Therefore, we have examined SWC-isolated tumor cells for the ability for increased retention of R123. Several reports have demonstrated this phenotype to be preferentially expressed in carcinoma cell lines but not in nonmalignant cultures (reviewed in Ref. 18). In one of the few instances of long-term culture of primary breast cancer (4), preferential retention of R123 was one of two markers used for the distinction between normal and tumor cells.

In the one case analyzed here more completely, the putative tumor cells derived from SWC display aneuploidy, thus providing additional evidence for malignant origin. The significance of this result lies in the fact that although a large majority of breast carcinomas contain aneuploid cell populations, cells cultured from these specimens under routine culture conditions are generally diploid and therefore not representative of the tumor as a whole (1, 2). Since DNA ploidy is an important prognostic marker for breast cancer, and patients with hyperdiploid tumors tend to have a poor prognosis (19), a culture setup for the routine isolation and expansion of aneuploid primary tumor cells provides the opportunity for new mechanistic insights and intervention modalities.

The tumor-derived SWC isolate, which was found to be aneuploid, also demonstrated a mutation in the p53 gene. The alteration observed in these cells is within one of the four hot spots containing the most frequent sites of missense mutations (20) and to our knowledge represents the first report of a primary breast tumor culture expressing a mutated p53 gene. We speculate that in the early stages of developing tumors, microenvironmental limitations of nutrient and oxygen supply, waste removal, low pH, etc., provide selective advantage to those cells that can override mechanisms of cell cycle arrest such as those induced by the p53 protein. In this regard, the SWC system, designed specifically to mimic this environmental milieu, may be particularly advantageous for the selection of cells harboring specific preexisting alterations such as a p53 gene mutation.

The tumor-derived isolates from sandwich culture are phenotypically distinct from normal cells in several ways, yet they become senescent unlike established cell lines isolated from metastatic specimens. They may therefore represent relatively early changes in malignant progression.

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References

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